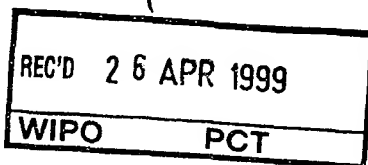


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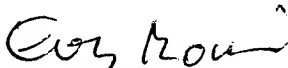
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Huvudföreläsningen

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NUCLEIC ACID TRANSFER

Technical field

5 The present invention relates to the transfer of genetic elements into the nucleus of a host cell by a novel specific and highly efficient method using a novel transfer conjugate. The invention also relates to advantageous uses of the novel conjugate according to the invention.

10 Background

Methods for genetic modification, wherein exogenous genetic material is introduced into host cells to provide function thereof, are usually limited by the rate of the uptake of the genetic material introduced into the cells. In eucaryotic cells, the nuclear uptake is often limiting. Even though direct injection methods have been used in this context, they are, however, extremely slow and labor-intensive. Thus, for use in larger scales, standard methods for transferring nucleic acids into cells are rather based on an uptake of complexes formed between different chemical compounds of nucleic acids. The genetic material is then entering the nuclei passively.

20 For example, Sebestyen *et al.* (Nat. Biotechnol., 1998, Jan;16:(1):80-85) have used digitonin permeabilized cells to enable nuclear translocation after chemically linking a nuclear localisation signal (NLS) peptide to a plasmid and they have injected it into the cytoplasm.

25 Yoneda *et al.* (Exp. Cell Res. 201:213 (1992)) have reported translocation of proteins larger than 970 kDa into the nucleus.

Summary of the present invention

30 The present invention solves the problems defined above by providing a novel conjugate, which enables an effective transfer of genetic material to a host cell, which

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may then be genetically modified by said transfer. The novel conjugate provides for such a transfer to take place with efficacy and efficiency, both of which are superior compared to the methods of the prior art. Further, the invention also provides a novel complex, which comprises the conjugate according to the invention associated with a suitable vector, as well as novel methods of transfecting eucaryotic cells. The present invention is especially advantageously used in gene therapy methods.

Brief description of the drawings

Figure 1 illustrates the principle of PNA-NLS interaction with a target site and subsequent nuclear translocation.

Figure 2 illustrates how this complex is subsequently transported into the nucleus.

Figure 3a shows translocation of fluorescently labeled oligonucleotides, while Figure 3b provides a graph, wherein the nuclear accumulation of the antisense oligonucleotide when compared to the sense oligonucleotide is shown.

Figure 4 illustrates that the increase of transfected cells is dependent on the purity of the PNA-NLS/plasmid complex.

Definitions

In the present application, the following terms and abbreviations are used as follows:

The terms "protein", "polypeptide" and "peptide" are used interchangeably herein and relates to any shorter or longer polymer of amino acid residues. In addition to naturally occurring amino acid polymers, the term also applies to amino acid polymers, wherein one or more amino acid residues are artificial chemical analogues of the corresponding naturally occurring amino acids.

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"PNA" refers to a Peptide Nucleic Acid and more specifically a DNA mimic with a pseudopeptide backbone consisting of aminoethyl glycine units, to which the nucleobases are attached via methylene carbonyl linkers. (See e.g. Nielsen, P.E.: Peptide nucleic acid (PNA): "A lead for gene therapeutic drugs", *Perspectives in Drug Discovery and Design*, Vol. 4, pp. 76-84; and Dueholm et al., *New J. Chem.*, 1997, 21, 19-31: "Chemistry, properties and applications of PNA".)

"NLS" refers to a nuclear localization signal, which may be any protein that recognizes and binds specifically to residues on other proteins.

A "conjugate" refers to an assembly of two different elements, such as a peptide nucleic acid and a protein.

"Transfection" is used herein as a general term for any uptake by a cell of genetic material from the culture medium.

The term "vector" is used herein to denote any plasmid, oligonucleotide, virus or other molecule or construct capable of transferring a nucleic acid into a cell.

A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunological or chemical means.

The term "hybridize" refers herein to any binding, duplexing or hybridization by base pairing of complementary bases of nucleic acids or peptide nucleic acids.

"Homologous recombination" refers to a method, wherein a nucleotide, e.g. a DNA, exchanges its sequence to another sequence, which is essentially or fully homologous. This technique is known to the skilled in this field. (For a review, see e.g. Cole Strauss, a., et al. in *Science*, vol. 273, & Sept., 1996: "Correction of the muta-

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tion Responsible for Sickle Cell Anemia by an RNA-DNA Oligonucleotide" and the references cited therein.)

5 As used herein, a "targeting sequence" is a sequence, which permits legitimate homologous recombination into the genome of the selected cell containing the gene of interest. The targeting sequence(s) used are selected with reference to the site into which a nucleotide, preferably a DNA, is to be inserted.

10 As used herein, "homologous" sequences are sequences, which are identical or sufficiently similar to cellular DNA such that the targeting sequence and cellular DNA can undergo homologous recombination.

15 The term "host cell" is used herein to denote any cell, wherein any foreign or exogenous genetic material has been introduced. In its broadest sense, "host cell" is used to denote a cell which has been genetically manipulated.

20 In the present context, a "transforming sequence" relates to any sequence that participates in a genetic modification event in a host cell, and may *e.g.* be a protein coding sequence, regulatory elements, an entire gene *etc.*

25 The term "recombinant" when referring to a cell is used herein simply to denote that a genetic modification has occurred therein. More specifically, it is used to indicate that a modification thereof have been obtained by the introduction of an exogenous nucleic acid, by the alteration of a native nucleic acid or that the cell is derived from a cell so modified.

Detailed description of the invention

30 More specifically, in a first aspect, the present invention relates to a novel conjugate comprised of a peptide nucleic acid (PNA) domain and a nuclear localization signal (NLS) domain. The novel conjugate according to the invention is particularly sui-

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table for use in methods aimed at transfecting eucaryotic cells and thus directing nucleic acids to the nucleus of the cell. However, depending on the particular PNA's and NLS's chosen in each case, the present conjugate may be designed to enable an effective and specific transformation of any cell with any gene or nucleotide sequence.

In a preferred embodiment thereof, the PNA-NLS conjugate according to the invention is described by the general formula

10 ...XXXXXXXX-OOOOOOO-NNNNNNNNN... (I),

wherein

X denotes a sequence of one or more PNA bases;

O denotes an non-reactive linker sequence; and

15 N denotes an NLS sequence.

The PNA domain is a synthetic DNA analogue, that binds strongly to DNA and RNA with a higher affinity than DNA-DNA, RNA-DNA or RNA-RNA binding. This sequence is designed in order to be specific for the nucleic acid to which it is intended to bind. PNA is metabolized very slowly and has also been shown to be non-toxic, which evidently is a great advantage when use in the medicinal field. In addition, PNA is capable of a highly specific binding to the sequence of the nucleic acid that is complementary thereto, which in turn provides a high frequency of correctly transformed cells.

25 In a particular embodiment, the linker sequence is essentially or completely uncharged. In this context, the term "non-reactive" is used to explain that the linker will not undergo any undesired or deleterious chemical reactions in the environment where it is used, e.g. that it does not react with any other components of the cell that it may contact during a transfection process according to the invention. Thus, it is

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also non-reactive as regards any reagents used in the present reaction as well as in view of the PNA and NLS domains. Said linker may *e.g.* be comprised of a polymer of a suitable number of amino acid residues, even though it is to be understood that any other molecule which functions as a spacer without interfering with the desired result may be used as linker in the conjugate according to the invention. The size and nature of the linker sequence is dependent on the surrounding PNA and NLS domains, as its function is to provide a sufficient spacing between said sequences to enable free interaction of the NLS with the transport protein that will carry the conjugate into the nucleus during the transfection process.

Preferably, the NLS sequence comprises a SV40 large T antigen protein or a fragment thereof, which exhibits the desired nuclear localizing signal properties. However, as the skilled in this field will realize, the choice of a suitable NLS sequence will depend on the intended future use of the present conjugate. Thus, the NLS may be of any other origin or composition, as long as it fulfills the desired functions of binding sufficiently strong and specific to a transport protein that will bring the conjugate into the nucleus of the host cell. The skilled in this field will be able to choose and produce the appropriate nuclear localization sequence for each case with reference of known techniques and methods disclosed in the literature.

In a specific embodiment, the PNA-NLS conjugate according to the invention is described by the formula:



wherein O is described by the formula:



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However, as the man skilled in the art will easily realize, variations may be made to these sequences while still providing an advantageous conjugate within the scope of the present invention as defined by the claims.

5 Thus, the PNA of the conjugate according to the invention provides excellent RNA and DNA hybridization properties and biological stability and is easily produced by solid phase peptide synthesis (see *e.g.* the international patent applications WO 95/01370 and WO 92/2072 and Nielsen, P.E.: Peptide nucleic acid (PNA): "A
10 lead for gene therapeutic drugs", *Perspectives in Drug Discovery and Design*, Vol. 4, pp. 76-84). A PNA-NLS conjugate according to the present invention is especially useful in the context of anti-sense constructs, since it may interfere with a RNA molecule already in the nucleus, thus blocking the possibility of protein expression completely.

15 A second aspect of the present invention is a complex suitable for transfection of eucaryotic cells, which complex comprises a PNA-NLS conjugate as described above and a vector, such as a plasmid, an oligonucleotide, such as a chimeric DNA-RNA construct, or the like. Said conjugate and vector are attached to each other by hybridization of the PNA domain of the conjugate to a PNA target sequence on the
20 vector. Such a hybridization is easily performed by someone skilled in this field. (Hybridization techniques are for example generally described in "*Nucleic Acid Hybridization, A Practical Approach*", Ed. Hames, B.D., and Higgins, B.D., IRL Press (1985); Gall and Pardue; *Proc. Natl. Acad. Sci. USA* 63: 378-383 (1969); and John *et al.*, *Nature* 223:582-587 (1969)). Oligonucleotides may be prepared by any
25 suitable method known to the skilled in this field, *e.g.* by direct chemical synthesis, such as the phosphotriester method of Narang *et al.*, *Meth. Enzymol.* 68:90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68:109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.*, 22:1859-1862 (1981); and the solid support method of US Patent no. 4 458 066.

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Preferably, the vector also carries one or more transforming sequences, such as protein coding sequences if the vector is a plasmid. Different vectors and the properties thereof are well known in the art and easily chosen by someone skilled. (For a general reference to laboratory procedures that may be used, see e.g. Sambrook et al.,
5 *Molecular Cloning, A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.)

10 In the preferred embodiment of the complex according to the invention, the vector also includes a marker or label, such as a fluorescent label, etc., to enable detection and identification of the cells that have included the complex. In case of a plasmid vector, the preferred label is a gene encoding a fluorescent protein, such as a green fluorescent protein (GFP). In case of an oligonucleotide vector, the marker is e.g. a fluorescent marker, such as Cy-3. Labelling and the detection thereof are well
15 known to the skilled in this field and are e.g. disclosed in US patents nos. 3 817 837; 3 850 752; 3 939 350; 3 996 345; 4 277 437; 4 274 149; and 4 366 241.

20 With the complex according to the invention, any transforming sequences may be introduced efficiently into a host cell due to the NLS ability to enter the nucleus thereof. Preferably, a genetic element capable of binding specifically to a predetermined site within the host cell is included in the vector to enable for a genetic modification to occur. Such a transformation may thus e.g. be provided by homologous recombination. The transfection complex according to the invention with its advantageous combined capability of efficient and specific transfer of genetic elements is of great value in many different applications, some of which will be disclosed in
25 more detail below.

Another aspect of the present invention is a method of transforming a eucaryotic cell, which method comprises the steps f

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(a) providing a transfection complex according to the invention by hybridizing a vector comprising at least one PNA target to the PNA domain of a PNA-NLS conjugate;

5 (b) contacting the complex formed in step (a) with the cell which is to be transformed in the presence of a transfection reagent;

(c) allowing the complex to enter the the nucleus of the cell; and

10 (d) allowing genetic transformation to take place, wherein the PNA conjugate is used as a the nuclear translocation initiator. Thus, any transforming sequences, which previously have been included in the vector on the requisite locations, may be transferred efficiently due to the NLS ability to enter the nucleus of the cell and the PNA ability to specifically bind to any vector which includes at least one PNA target sequence.

15 As regards step (a), a variety of hybridization formats is known to those skilled in this field, such as sandwich assays and competition or displacement assays. Hybridization techniques are for example generally described in "*Nucleic Acid Hybridization, A Practical Approach*", Ed. Hames, B.D., and Higgins, B.D., IRL Press (1985); Gall and Pardue; *Proc. Natl. Acad. Sci. USA* 63: 378-383 (1969); and John *et al.*, *Nature* 223:582-587 (1969).

20

Accordingly, firstly, the present method utilizes the ability of the NLS to bind to a transport protein, whereby it is brought into the nucleus of the host cell together with any elements bound thereto, which in the present case is the PNA and preferably a vector bound thereto. Once inside the nucleus, the NLS-protein complex will
25 be dissolved and the transport protein will exit the nucleus while the transported PNA-NLS conjugate will remain therein. Thus, secondly, specific regions of the vector attached to the conjugate according to the invention will be utilized to perform a transformation of the host cell. Meanwhile, the transport protein will be able to repeat its function by carrying more conjugates into the nucleus thus contributing

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to the surprisingly high transformation frequency obtained by use of the present transfection complex when compared to the prior art techniques.

Thus, even though Sebestyen *et al.* as mentioned above have used a nuclear localisation signal for genetical modification of cells, they used a method of injection instead of passive transfection, they used digitonin instead of the lipid reagent used according to the present invention, and importantly, no PNA was used to enable the advantageous inclusion of further elements. Thus, there are essential differences in methods between Sebestyen *et al.* and the present invention, which is the explanation to the substantial differences in transformation frequency obtained.

Thus, in a preferred embodiment, the transfection reagent is a polymer transfection reagent, such as PEI (polyethylene imine). PEI has previously been used in gene therapy experimental set-ups and has been reported to be non-toxic as well as capable of providing a high transfection efficiency. The pathway for PEI transfection is different from the standard lipid based transfection reagents commonly used today. The polymer functions as a proton acceptor and is believed to disrupt the endosomes by osmotic stress, thus releasing nucleic acids.

In a specific embodiment of the invention, the method according to the invention further comprises an additional step (e), wherein the resulting transformation is confirmed by measuring a previously included label or marker. The nature and identity of such labels and markers are also discussed elsewhere in this application.

In one advantageous embodiment of the method according to the invention, the transformation defined in step (d) introduces one or more protein coding sequences by use of a plasmid vector. Thereby, an efficient transformation yielding a host cell expressing an exogenous, or non-native, protein or polypeptide is obtained. In an alternative embodiment of the invention, the transformation according to step (d) may be used to introduce one or more gene regulatory sequences in the host cell,

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whereby an otherwise silent gene may be expressed. This latter embodiment uses a technique known as gene activation, which is described in detail *e.g.* in US patent no. 5 641 670. In either one of the two above disclosed embodiments of the present method, the resulting transformed cell may be used to produce substances such as proteins useful as medicaments.

In a further embodiment of the present method, the transformation defined in step (d) is aimed at repairing a mutation in the host cell, which *e.g.* may be obtained by homologous recombination. In an alternative embodiment, said transformation is aimed at introducing a mutation in a host cell. This may be desired, *e.g.* if the expression of a gene is not desired due to the nature of the expression product or in the production of animal models for the study of various genetic diseases.

A further advantage of the present invention is that the PNA-NLS conjugate has a broad applicability and therefore a small number of conjugates may be used to transport more than 90% of all the plasmids conventionally used on an everyday basis in research laboratories worldwide.

Accordingly, another aspect of the present invention is a recombinant cell produced by a method as disclosed above. The invention also relates to animal models, such as mice, produced by a method according to the invention specifically designed for the study of certain genomic defects. General cloning techniques and methods of culturing cells are well known to someone skilled in this field. (See *e.g.* Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989; Freshney: *Culture of Animal Cells, A Manual of Basic Technique*, 3rd ed., Wiley-Liss, New York, NY (1994)).

One particularly advantageous aspect of the present invention is the use of the PNA-NLS conjugate and the transfection method disclosed above in gene therapy. For such an application, the conjugate may be hybridized to an oligonucleotide vector,

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such as a chimeric DNA-RNA construct. Gene therapy procedures have been used to correct acquired and inherited genetic defects in a number of contexts. The ability to express artificial genes in humans, or animals, such as mammals, facilitates the prevention and/or cure of many important diseases, often not amenable to treatment with other therapies. However, presently available approaches to gene therapy make use of infectious vectors, such as retroviral vectors, which include the genetic material to be expressed. Such approaches have limitations, such as the potential of generating replication-competent virus during vector production; recombination between the therapeutic virus and endogenous retroviral genomes, potentially generating infectious agents with novel cell specificities; host ranges, or increased virulence and cytotoxicity; independent integration into large numbers of cells, increasing the risk of tumorigenic insertional event; limited cloning capacity in the retrovirus (which restricts therapeutic applicability) and short-lived *in vivo* expression of the product of interest. Thus, the use according to the present invention, wherein the PNA-NLS conjugates or complexes according to the invention are used, avoids the limitations and risks associated with the virus methods of the prior art. For example, previously, in the context of cystic fibrosis, adenovirus vectors have been used as a vector in gene therapy. Such a vector may give rise to undesired and harmful immunological responses, which accordingly will be avoided by the advantageous use of the novel PNA-NLS conjugate according to the invention. Preferably, a transfection reagent as disclosed above is used.

Consequently, the invention also relates to gene therapy methods as such, wherein conjugates or complexes according to the invention are used, preferably together with at least one transfection reagent, preferably a lipid reagent, such as the above described polyethylene imine (PEI). Such methods are often aimed at repairing a mutated or defect gene, but may also be utilized to introduce a mutation, e.g. to prevent the expression of an undesired protein or to produce an animal model for the study of a certain defect. One specific example of a disease that may be treated by gene therapy is cystic fibrosis, CF, which afflicts 1/10 000 people and where about

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70% of the afflicted patients exhibit a point mutation in the CFTR gene. CF would be especially suitable for plasmid mediated gene transfer, as the target organ is the lung which is easily accessible. Of the same reasons, CF is also suitable for performing homologous recombination. However, as the number of diseases for which a genetic defect is identified steadily increases, it is predicted that in the future, additional gene related conditions or sicknesses will be identified as highly suitable for treatment by gene therapy according to the present invention. (For a general review of gene therapy methods, see e.g. Anderson, *Science* (1992) 256:808-813; Nabel and Felgner (1993) TIBTECH 11: 211-217; Mitani and Caskey (1993) TIBTECH 11:162-166; Mulligan (1993) *Science* 926-932; Dillon (1993) TIBTECH 11:167-175; Miller (1992) *Nature* 357:455-460; Van Brundt (1988) *Biotechnology* 6(10):1149-1154; Vigne (1995) *Restorative Neurology and Neuroscience* 8: 35-36; Kremer and Perricaudet (1995) *British Medical Bulletin* 51(1) 31-44; Haddada et al. (1995) in *Current Topics in Microbiology and Immunology*; and Yu et al., *Gene Therapy* (1994) 1:13-26.

A similar aspect of the present invention is the use of a PNA-NLS conjugate or complex according to the invention to genetically modify cells to be used in cell therapy. (For a disclosure of the fundamentals of cell therapy methods, see e.g. Gage, F.H., *Nature*, vol. 392, 30 April 1998.) Consequently, the invention also relates to such cell therapy methods as well as to cells used therein that have been genetically modified by a method according to the present invention.

Detailed description of the drawings

Figure 1 illustrates the principle of PNA-NLS interaction with a target site and subsequent nuclear translocation. The antisense oligonucleotide hybridizes to the sense PNA-NLS.

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Figure 2 illustrates how this complex is subsequently transported into the nucleus. The plasmid attracts a high local concentration of PNA-NLS which is enough to confirm nuclear translocation.

- 5 Figure 3a shows translocation of fluorescently labeled oligonucleotides. The antisense oligonucleotide is Cy-5 labeled. The sense oligonucleotide is Cy-3 labeled. As can be seen, the Cy-5 oligo dominates in the nucleus showing a high specific transport of the antisense PNA-NLS complex.

- 10 Figure 3b provides a graph, wherein the nuclear accumulation of the antisense oligonucleotide when compared to the sense oligonucleotide is shown.

- 15 Figure 4 illustrates that the increase of transfected cells is dependent on the purity of the PNA-NLS/plasmid complex. Since a certain amount of free PNA-NLS is present, it will block the nuclear translocation unless separated from the transfection mix.

EXPERIMENTAL

- 20 Below, the present invention will be further disclosed by way of examples. It is to be understood that the examples are merely illustrating the invention and are not to be construed as limiting the scope as defined by the appended claims. All references below and elsewhere of this applications are hereby included herein by reference.

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Materials and methods:**Cell lines and medium**

COS-7 cells were used for transfections and cultivated in DMEM, 4500 mg/l glucose and 50 µg/ml gentamicine.

The synthetic DNA oligo, peptide nucleic acid, were synthesised at Perseptive Bio-Synthesis Ltd. The sequence of PNA was selected so that it would not interfere with future sequences regarding plasmid transfections, GCGCTCGGCCCTTCC. The PNA oligo was linked to a stretch of amino acid residues, PKKKRKV, the SV40 core NLS. The complete sequence selected were in two versions:

A.GCGCTCGGCCCTTCC-linker-PKKKRKV or

B.PKKKRKV-linker-GCGCTCGGCCCTTCC. Only the A type has worked in nuclear translocation so far.

Fluorochrome labelled oligonucleotides

Two fluorochrome labelled oligonucleotides were synthesised at Cybergene AB. One antisense Cy-5 labelled, AS, is complementary to the PNA-NLS hybrid. The other is sense Cy-3 labelled, S, is not complementary to the PNA-NLS hybrid.

Transfections with PNA-NLS and fluorochrome labelled oligonucleotides

Transfections were made with 25 kD PEI as follows. PNA-NLS:AS:S were mixed at a molar ratio of 1:1:1 and heated to 90° C. The mix was allowed to slowly cool to room temperature to allow for optimal hybridization of PNA-NLS to the As-oligo. The mix was diluted to a concentration of 0,05 µg/µl. To the mix of oligonucleotides and PNA-NLS, 1.44 µl of 0.1 M 25 kD PEI solution was added/2µg nucleotide. The transfection solution was allowed to form complexes in room temperature for 10 minutes and was subsequently mixed with 1 ml DMEM with 10% bovine serum and 100 µg/ml gentamicin.

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For transfection 100 000 COS-7 cells were plated per well in 6-well plates with 2 ml medium per well 24h prior to the transfection. Incubation time for oligonucleotide transfections were 10h. All incubations were made at 37°C in 5% CO₂.

5 Transfections with PNA-NLS and EGFP plasmid

The plasmid pEGFP-N3 (Enhanced Green Fluorescent Protein (Clontech)) was modified by use of standard technique to include the target sequence for the PNA-NLS hybrid. The pEGFP-N3 plasmid was digested with Afl II and ligated with a oligonucleotide fragment containing the PNA target sequence flanked with Afl II sites. Different clones of the construct were used containing different numbers of PNA target sites. This was of importance since it has been reported that up to 8 sequential NLS signals can improve the nuclear uptake (ref.). Based on these reports a plasmid clone with 11 sequential PNA target sites was chosen. The expression from the EGFP gene gave information about the status of the plasmid in respect to functionality of the EGFP gene and by directly scoring the number of GFP positive cells the enhanced nuclear uptake was verified. Transfections were made with 25 kD PEI as follows. PNA-NLS:plasmid were mixed at a molar ratio of 1:1 and heated to 95°C. The mix was allowed to slowly cool to room temperature to allow for optimal hybridization of PNA-NLS conjugate according to the invention to the plasmid target site. The mix was diluted to a concentration of 0.05 µg/µl. To the mix, 1.44 µl of 0.1 M 25 kD PEI solution was added. The transfection solution was allowed to form complexes in room temperature for 10 minutes and was subsequently mixed with 1 ml DMEM with 10% bovine serum and 100 µl/ml gentamicin.

For transfection 100 000 COS-7 cells were plated per well in 6-well plates with 2 ml medium per well 24h prior to the transfection. Incubation time for oligonucleotide transfections were 48h for the plasmid transfections. All incubations were made at 37°C in 5% CO₂.

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Fluorescence microscopy and image analysis

Fluorescence microscopy was performed on a Leica DXM2 microscope with a cooled frame CCD (Coupled Charge Device) camera. The subsequent image analysis was performed with the Software Slidebook 2.1.4 from Intelligent Imaging Ltd.

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Results

PNA hybridized oligonucleotides

The experiment with the fluorochrome labelled oligonucleotides clearly shows an increased nuclear translocation of oligonucleotide hybridized to the PNA-NLS conjugate of the invention by 200-800% (Fig. 3). The increased nuclear uptake was calculated by masking the nucleus and then measuring the fluorescence from the Cy-3 and the Cy-5 spectra, respectively, and subtracting the background. The levels of accumulated oligonucleotides in different cellular localizations clearly shows that the system for nuclear localization based on the SV40 NLS is saturated (Fig. 2a).

15

PNA hybridized plasmids

Experiments with lacZ PNA-target plasmid transfections shows a 3-6 fold increase in transfection efficiency when the plasmid was hybridized with the PNA-NLS conjugate according to the invention (data not shown). The transfection experiments with pEGFP-N3 PNA plasmid/fragment shows 6-8 fold transfection efficacy increase (see figures). The efficiency of gene transfer was measured via the frequency of GFP expressing cells. The transfection efficacy was enhanced when the PNA-NLS-plasmid complex according to the invention was purged from free PNA-NLS, that otherwise blocked the nuclear transport of the plasmid. When mixing with a control plasmid with PNA-NLS, no effect could be seen on transfection efficacy.

25

Discussion

The conjugate according to the present invention can work as a nuclear targeting signal when hybridized to a fluorescent labeled oligonucleotide or a plasmid with a reporter gene. The new technique according to the present invention is of potential

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great value for general transfections and for specific transfections in the context of gene therapy. The increased uptake of nucleic acids into target cells is vital for gene expression, expression from anti-sense constructs and the function of targeted mutation repair (for targeted mutation repair, see Cole-Strauss *et al.*, *Science* 273:1386-1389 (1996)). In the context of anti-sense constructs, the use of a PNA-NLS conjugate according to the invention is foreseen. The use of a PNA target sequence, CGC GAG CCG GGA AGG, that does not exist in nature, is disclosed herein. The EGFP plasmid or the LacZ plasmid were also used in the experiments. The SV40 sequence was chosen simply because it has been the most studied NLS, which however does not restrict the present invention to that particular sequence. The discrepancies between the lacZ and EGFP experiments is due to unsatisfactory purification of unbound PNA-NLS in the lacZ experiment. In the case of the EGFP, the complex was purified extensively on a PCR purification column (Quiagen). The PNA-NLS system according to the invention is predicted to be efficient in assays and therapies involving a transient treatment as well as in systems where repair constructs are shuttled into the nucleus for homologous recombination. The system for nuclear translocation based on the SV40 NLS was saturated (Fig. 2). This is because different cytoplasmic levels still give rise to the same nuclear levels of the targeted oligo, while the non-targeted oligo shows a relatively broad variation of nuclear levels. To further enhance the nuclear import of oligonucleotides a mix of PNA-NLS conjugates may be used, in which the NLS domain of the conjugates are preferably of different NLS pathways, so that one pathway will not limit the nuclear translocation of the transfected molecules. Examples of each group to target are the following NLS receptors: Qip1, Rch1 and NPI-1 (Yoichi Miyamoto *et al.*, *J. Biol. Chem.*, vol. 272, No. 42, Oct. 17, pp. 26375-26381, 1997). A way to mimic the lentivirus nuclear entry would be to bind the HIV-1 Matrix Association (MA) protein to the nucleic acid construct via a PNA-NLS conjugate according to the invention, where the DNA domain confers the binding site for MA. The complex would bind Vpr and in this form be transfected into the target cell and this way mimic the HIV-1 Pre-Integration Complex, PIC. The function of the MA-Vpr complex is similar to the

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extended SV40 NLS sequence described by Chong-Yun *et al.* (Chong-Yun Xiao, Stefan Hubner and David A. Jans. J. Of Biol. Chem 272 (August 29, 1997):35, p. 22191-22198) where they describe how the inclusion of a longer sequence of the SV40 NLS can alleviate the nuclear import 50-fold. The sequence,

- 5 SSDDEATADSQHSTPPKKRKV, contains a CKII site, a dsDNA-PK site, a sds2 site and the core SV40 NLS sequence. According to Chong-Yun *et al.*, phosphorylation occurs of the CKII and dsDNA-PK sites that confirms a large affinity increase in these sites for importin-58. This stabilisation is analogous to the HIV-1 PIC, where MA-Vpr complex stabilises the karyopherin α to ensure high levels of transport
- 10 into the nucleus (Michael I. Bukrinsky and Omar K. Haffar, Molecular Medicine 4:138-143, 1998). PNA-linked carriers will be used to mimic these functions. In this fashion, the complexes may be directed anywhere over the entire nucleic acid without disturbing the normal functions.

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CLAIMS

1. A conjugate suitable for use in genetic transformation of eucaryotic cells, which comprises one peptide nucleic acid (PNA) domain and one nuclear localisation signal (NLS) domain.

2. A PNA-NLS conjugate according to claim 1 described by the general formula

...XXXXXXXX-OOOOOOO-NNNNNNNN... (1),

wherein

X denotes a sequence of one or more PNA bases;

O denotes an linker sequence; and

N denotes an NLS sequence.

3. A PNA-NLS conjugate according to claim 2, wherein the linker sequence is essentially uncharged.

4. A PNA-NLS conjugate according to any one of claims 1-3, wherein the NLS sequence comprises a SV40 large T antigen protein or a fragment thereof exhibiting nuclear localizing signal properties.

5. A transfection complex, which comprises a PNA-NLS conjugate according to any one of claims 1-4 and a vector, which vector comprises at least one PNA target domain capable of specific binding in the host genome as well as a transforming sequence, said conjugate being attached to the vector by hybridization between the PNA domain thereof and a PNA target sequence present on the vector.

6. A complex according to claim 5, wherein the vector is a plasmid further comprising at least one additional PNA targeting sequence.

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7. A complex according to claim 6, wherein the transforming sequence is a gene encoding a protein or any subsequence of such a gene.

5 8. A complex according to claim 5, wherein the vector is an oligonucleotide, such as a chimeric DNA-RNA oligonucleotide.

9. A complex according to claim 8, wherein the transforming sequence is a mutation repairing or mutation inducing sequence.

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10. A method of transforming a eucaryotic cell, which method comprises the steps (a) providing a PNA-NLS complex according to any one of claims 5-9 by hybridizing a vector, which comprises at least one PNA targeting sequence, to the PNA domain of a PNA-NLS conjugate according to any one of claims 1-4;

15 (b) contacting the complex formed in step (a) with the cell which is to be transformed in the presence of a transfection reagent;

(c) allowing the complex to enter the cell nucleus; and

(d) allowing genetic transformation to take place, wherein the PNA targeting sequences of the vector interacts with PNA targets in the host cell.

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11. A method according to claim 10, wherein the transfection reagent is a polymeric transfection reagent, such as polyethylene imine.

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12. A method according to claim 10 or 11, which further comprises a step (e) wherein the occurrence of the genetic transformation is confirmed.

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13. A method according to any one of claims 10-12, wherein the vector is a plasmid which further comprises a gene encoding a labelled protein for the confirmation of the genetic transformation, preferably a GFP gene.

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14. A method according to any one of claims 10-12, wherein the vector is an oligonucleotide which further comprises a marker for confirmation of the genetic transformation.

5 15. A recombinant cell which has been produced by a method according to any one of claims 10-14.

10 16. Use of a PNA-NLS conjugate according to any one of claims 1-4, a PNA-NLS conjugate according to any one of claims 5-9 or a recombinant cell according to claim 15 in gene therapy.

17. An assay comprising a PNA-NLS conjugate according to any one of claims 1-4 or a PNA-NLS complex according to any one of claims 5-9.

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ABSTRACT

The present invention relates to a conjugate suitable for use in genetic transformation of eucaryotic cells, which comprises one peptide nucleic acid (PNA) domain and one nuclear localisation signal (NLS) domain. More specifically, the PNA-NLS conjugate according to the invention may be described by the general formula

...XXXXXXXX-OOOOOOO-NNNNNNNN... (I),

wherein

10 X denotes a sequence of one or more PNA bases;

O denotes an linker sequence; and

N denotes an NLS sequence.

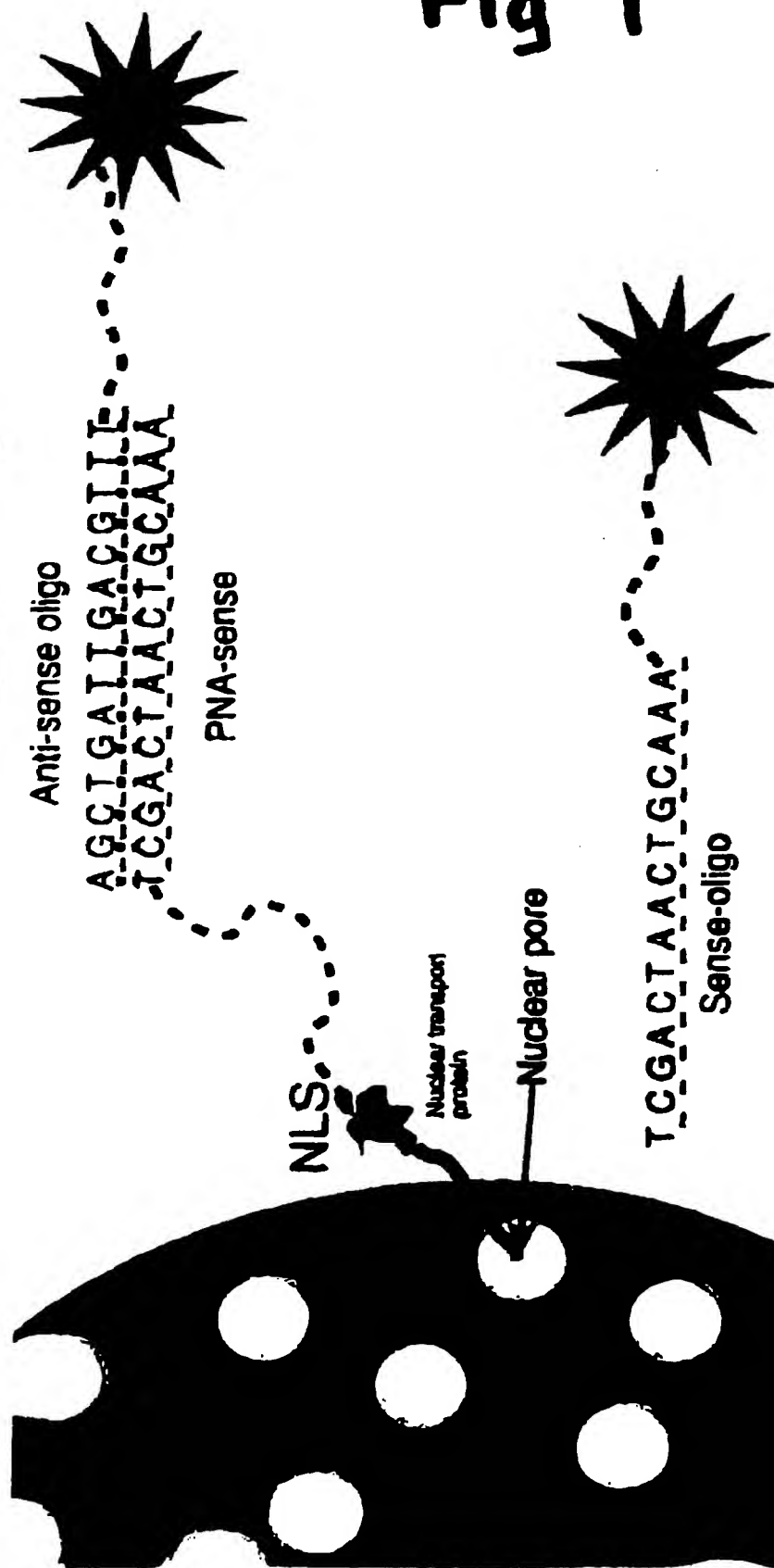
A further aspect of the present invention is a transcription complex comprised of a conjugate as described above associated with a vector, such as a plasmid or an oligonucleotide. In addition, the invention also relates to use of the conjugates and complexes as described above in the treatment of genetic diseases, preferably in gene therapy methods.

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Fig 1

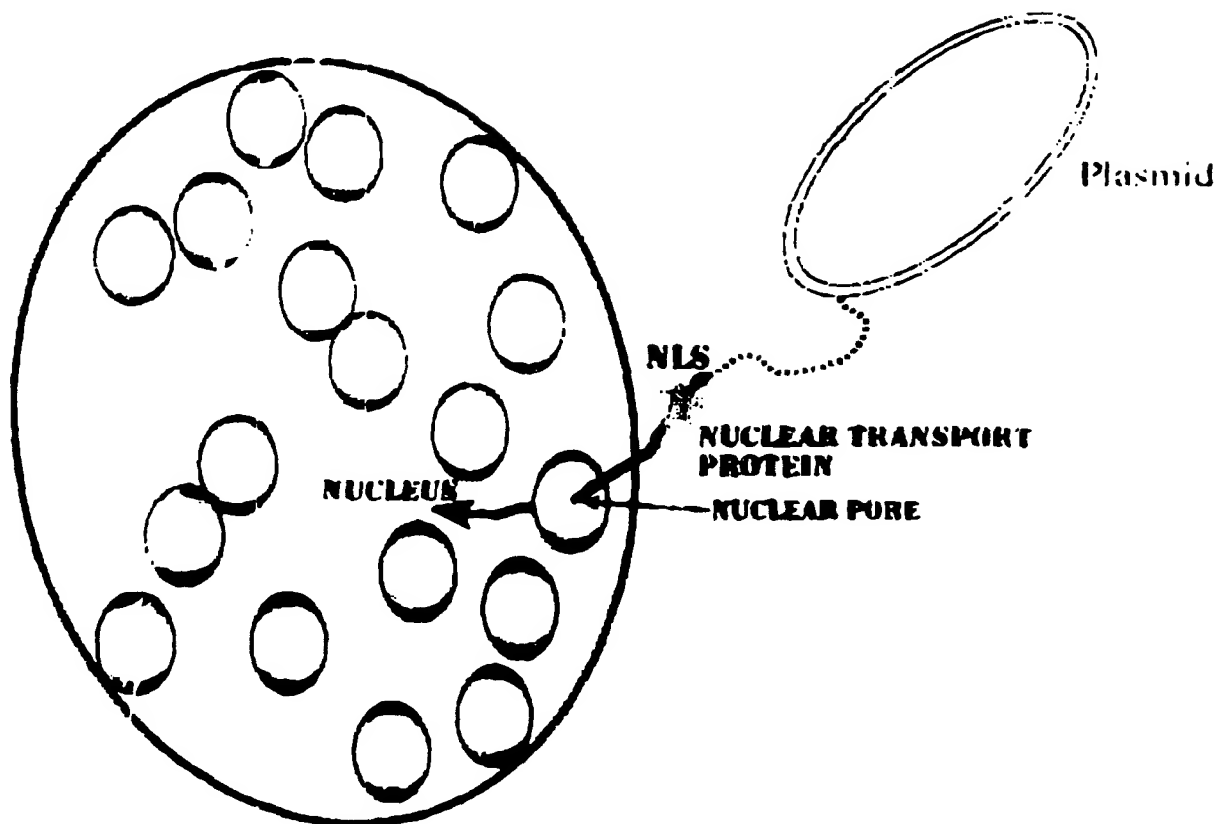


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Fig. 2

NUCLEAR TARGETING OF NUCLEIC ACIDS



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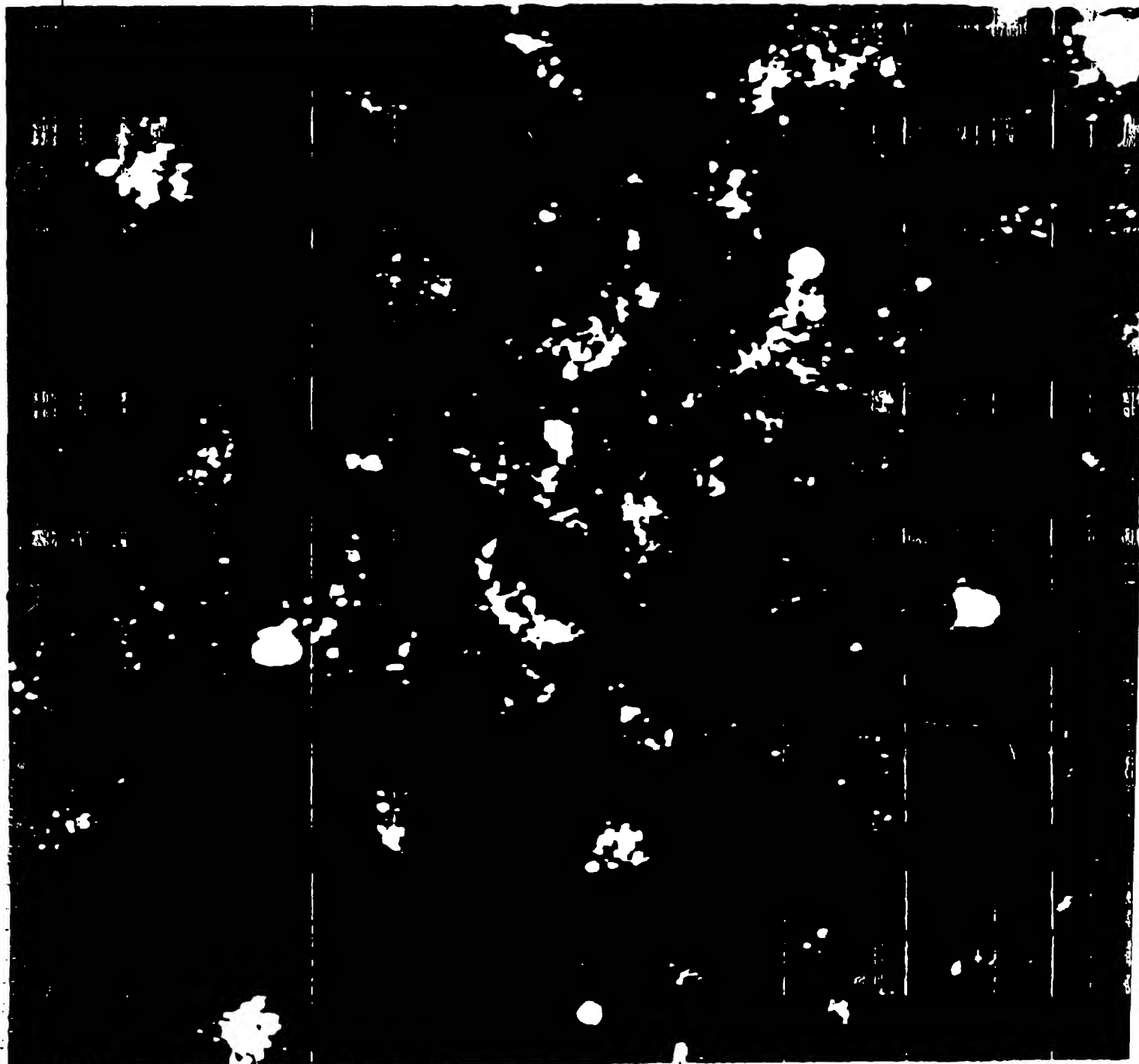
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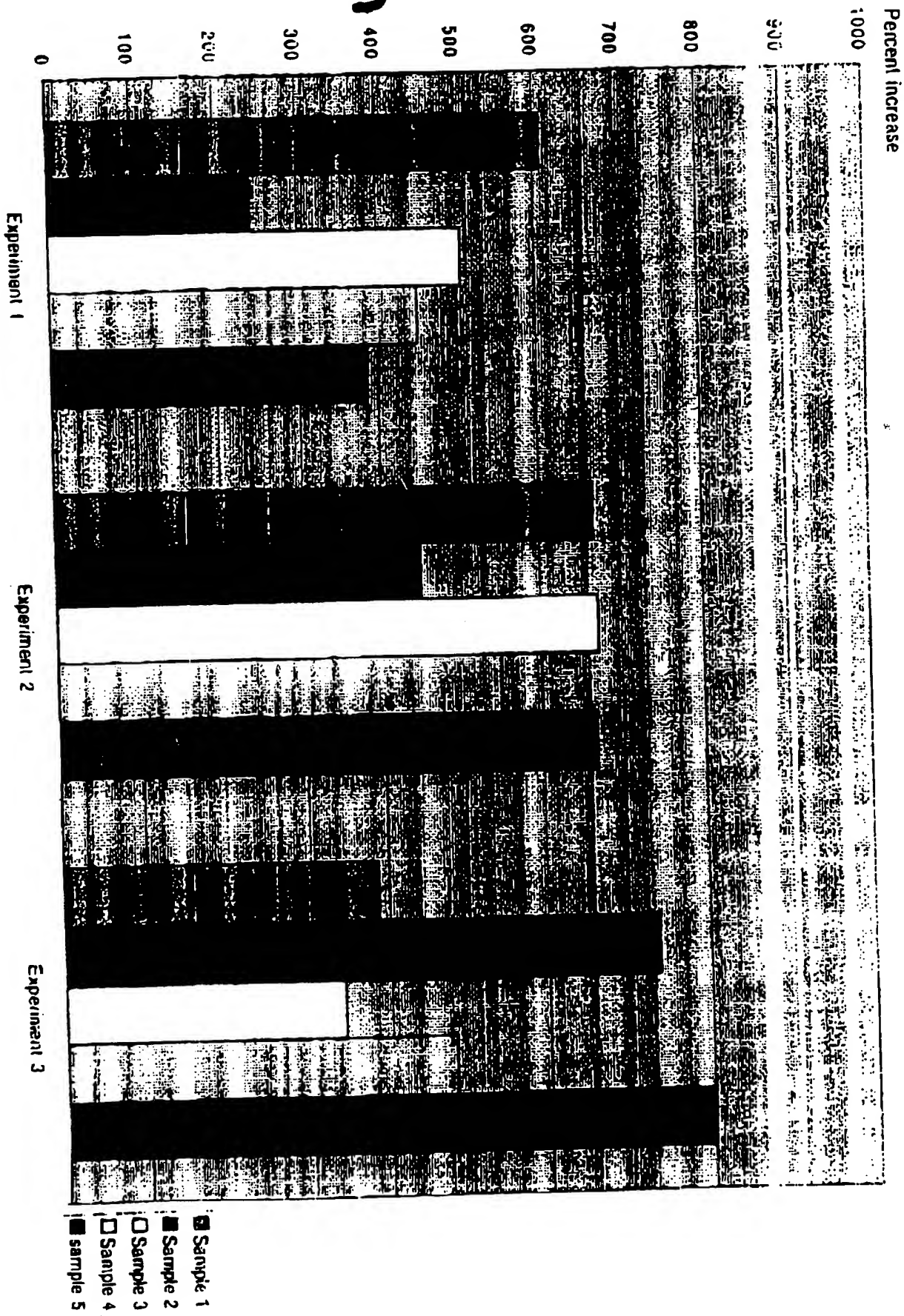
Fig. 3a



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Fig 3b



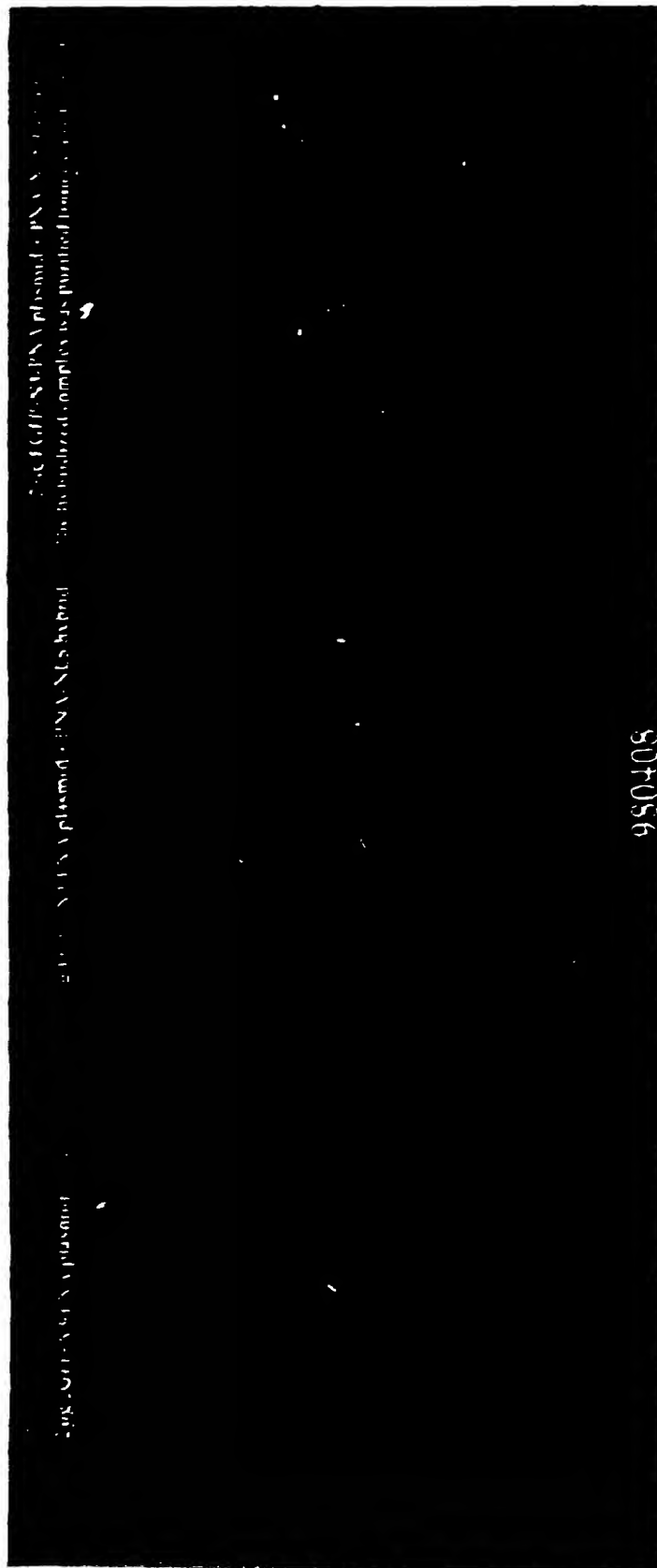
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Fig. 4



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